***New Phytologist* Supporting Information**

Article title: The Antiphasic Regulatory Module Comprising *CDF5* and its Antisense RNA *FLORE* Links the Circadian Clock to Photoperiodic Flowering

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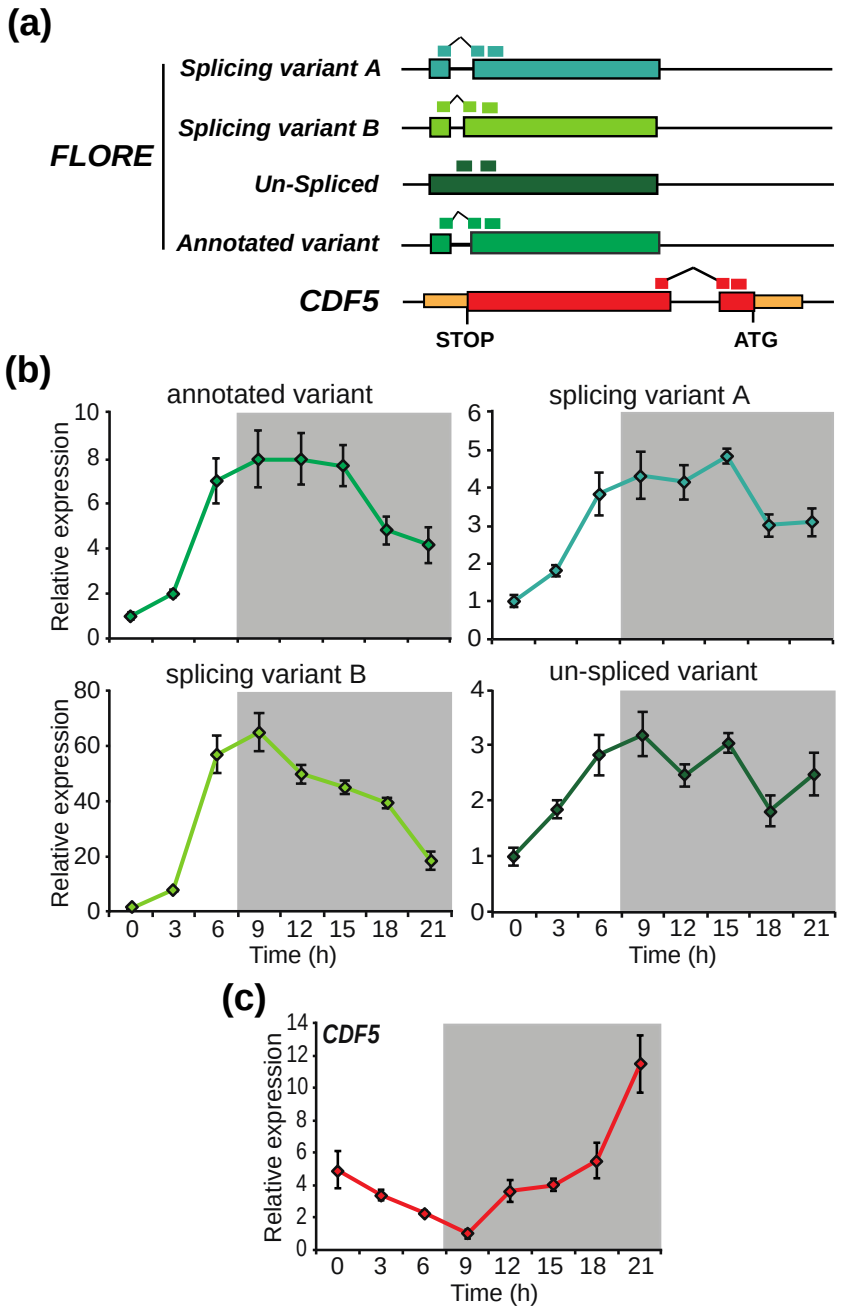


Fig. S1 Description of the *FLORE*/*CDF5* NAT pair under short day conditions. Cloning of the *FLORE* lncRNA revealed four different splicing variants; including TAIR 10 "other RNA" (At1g69572) here presented as "Annotated variant"; and three other variants where the reported intron is longer (A), shorter (B) or absent (Un-spliced) **(a)**. Nevertheless, regardless of the splicing event considered, all the variants had a similar expression pattern in WT grown under short day conditions (8h light / 16h dark) that is antiphasic to the *CDF5* waveform **(b, c)**.

Expression patterns were determined by qPCR after normalizing with *Actin2* and using specific primers to each splicing variant represented as squares above the schematic drawings in **(a)**. Values are means ± SD of three technical amplifications with one representative experiment shown out of three biological replicates tested. Grey rectangles correspond to the dark period. Time (h) represents the hours after lights on.

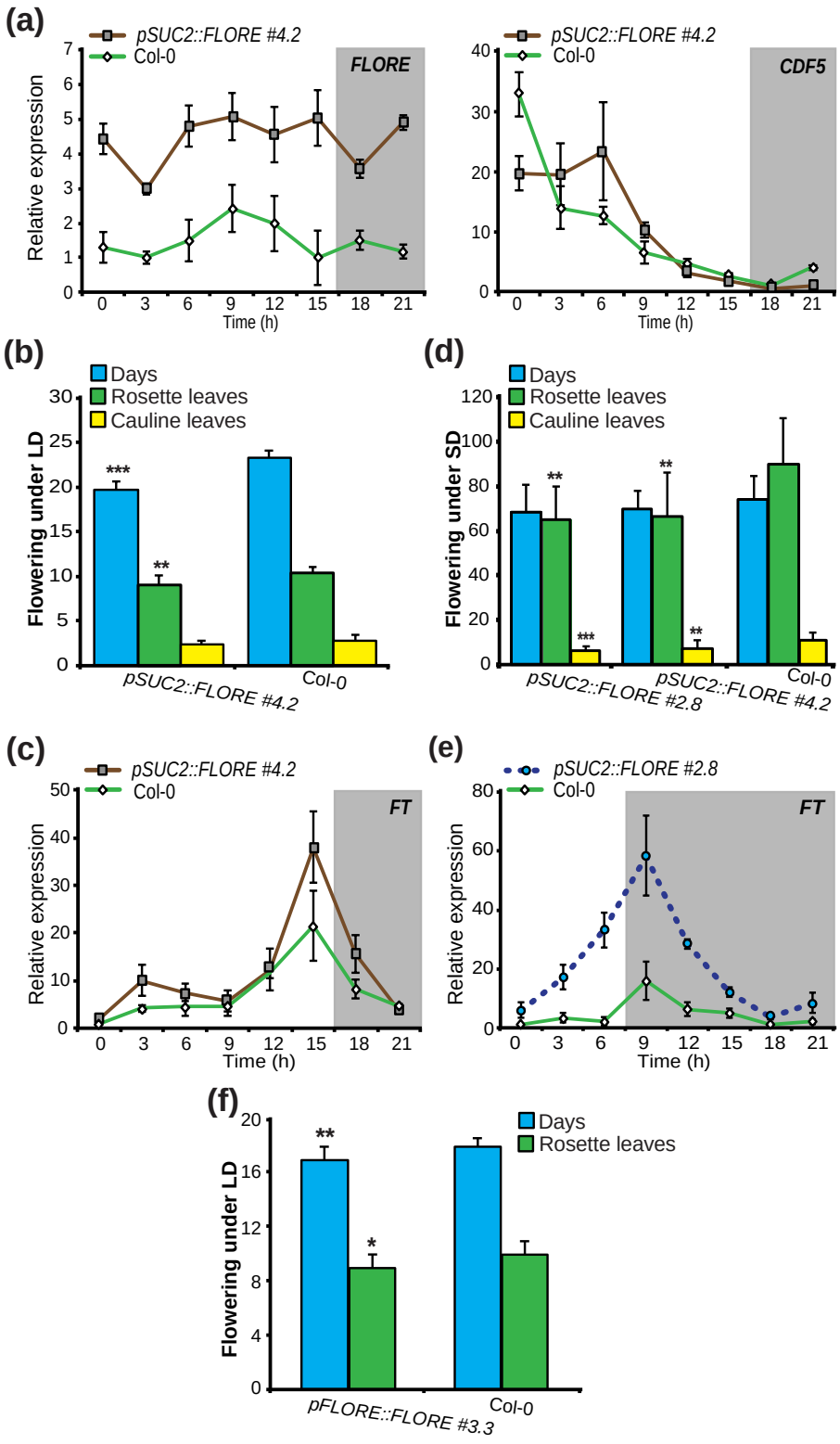


Fig. S2 *FLORE* vascular expression promotes early flowering both under long day and short day conditions. Vascular expression of *FLORE* in transgenic line *pSUC2::FLORE #4.2* is

lower that in *pSUC2::FLORE #2.8* (see Fig. 3a), although it also affects the *CDF5* waveform leading to a reduction in its amplitude and phase delay **(a)**. Grey rectangles represent the dark period. Transcript levels were determined by qPCR after normalization with *Actin2* and results shown are means ± SD of three technical replicates from one representative experiment out of two biological duplicates analysed. In addition, and similarly to line *pSUC2::FLORE #2.8*, vascular accumulation of *FLORE* in *pSUC2::FLORE #4.2* promotes early flowering under long days (LD) measured by number of days (blue), rosette (green) and cauline (yellow) leaves (Student's *t*-test, ***P<0.0001, **P<0.005) in two independent experiments (n=24) **(b)**. This phenotype directly correlates with the induction of *FT* expression **(c)** determined by qPCR performed as described above. Interestingly, the early flowering phenotype was also observed under short day conditions (SD) **(d)** in the two independent *pSUC2::FLORE* lines *#2.8* and *#4.2*, measured as number of rosette and cauline leaves (Student's *t*-test ***P<0.005, **P<0.05; n=20 and 19, respectively) in two biological replicates analysed. *FT* accumulation evaluated by qPCR was also observed under these conditions **(e)**. In addition, *FLORE* expressed under the control of its own promoter **(f)** also induced flowering under long day conditions (Student's *t*-test **P<0.01, *P<0.05; n=38). Shown is one representative experiment out of three biological replicates. Unless otherwise stated, each flowering experiment included ten wild type plants in each biological replicate.

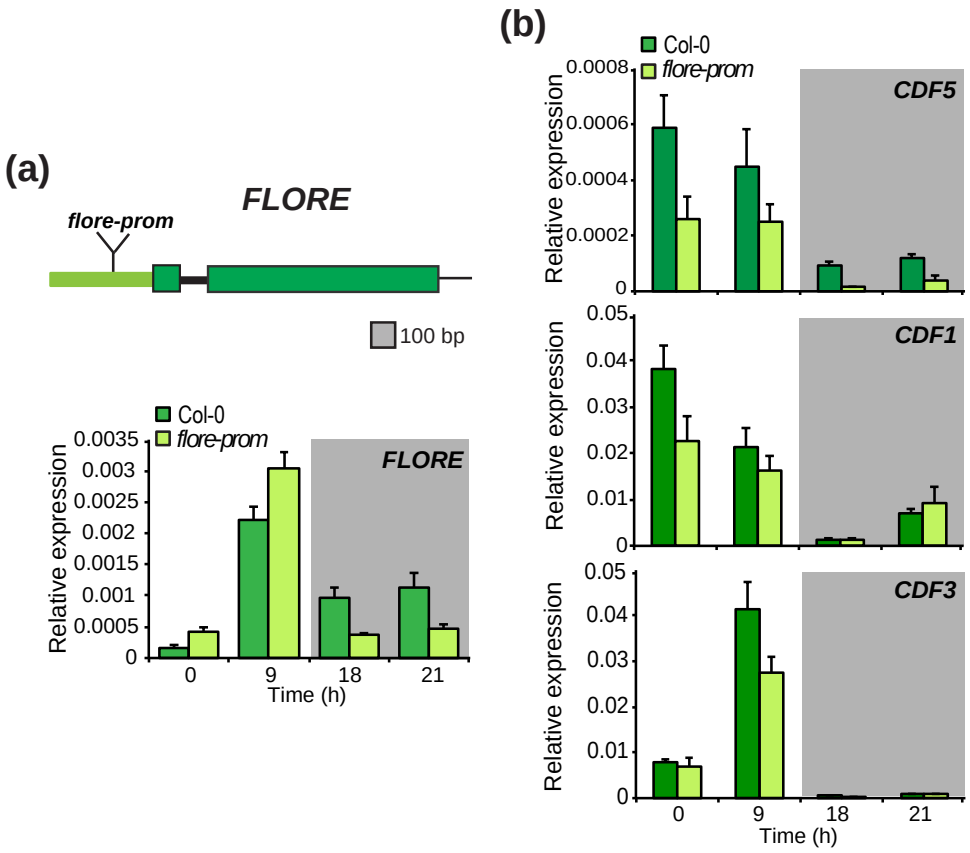


Fig. S3 Modulation of *FLORE* transcripts affects *CDF*s. (a) T-DNA insertion in the *FLORE* promoter region (*flore-prom*) differentially affects *FLORE* expression. *FLORE* transcript levels were determined by qPCR after normalization with *Actin2* using the $2^{-\Delta Ct}$ calculation method. Dark green rectangles represent exons, the thick black line depicts the intron and the light green rectangle indicates part of the *FLORE* promoter. Sizes are approximate. **(b)** The expression levels of *CDF5* (upper panel), *CDF1* (middle panel) and *CDF3* (lower panel) are altered in *flore-prom* mutants especially during the light period in seedlings grown under long day conditions, determined by qPCR after normalization with *Actin2*. Results shown are the mean ± SD of three technical replicates from one representative experiment out of two biological replicates analysed. Grey rectangles represent the dark period and Time (h) indicates the hours after lights on.

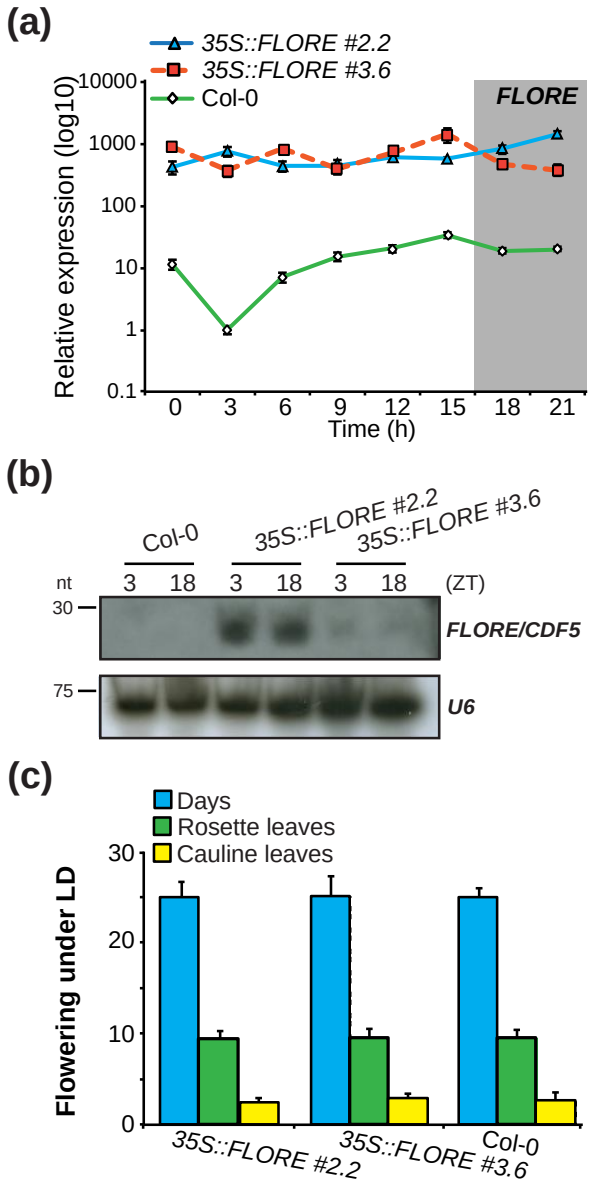


Fig. S4 *FLORE* biological function requires tissue specificity and is mostly independent of siRNA accumulation. *FLORE* constitutive expression under the *CaMV35S* promoter **(a)** induces siRNA accumulation, which is absent in wild type plants (Col-0) **(b)**. siRNA detection by small RNA Northern was repeated twice with similar results. However, siRNA accumulation is not mirrored in a change in flowering time in both *35S::FLORE #2.2* and *35S::FLORE #3.6* lines grown under long day conditions determined by three parameters; number of days (blue), rosette leaf (green) and cauline leaf numbers (yellow) **(c)**. Shown is a representative experiment out of

three biological replicates (LD; n=34 for both lines). *FLORE* transcript waveforms were determined by qPCR after normalization with *Actin2*. Values are means ± SD of three technical triplicates from one representative experiment out of two biological duplicates evaluated. Grey rectangles represent the dark period under long day conditions. Time (h) indicates the hours after lights on.

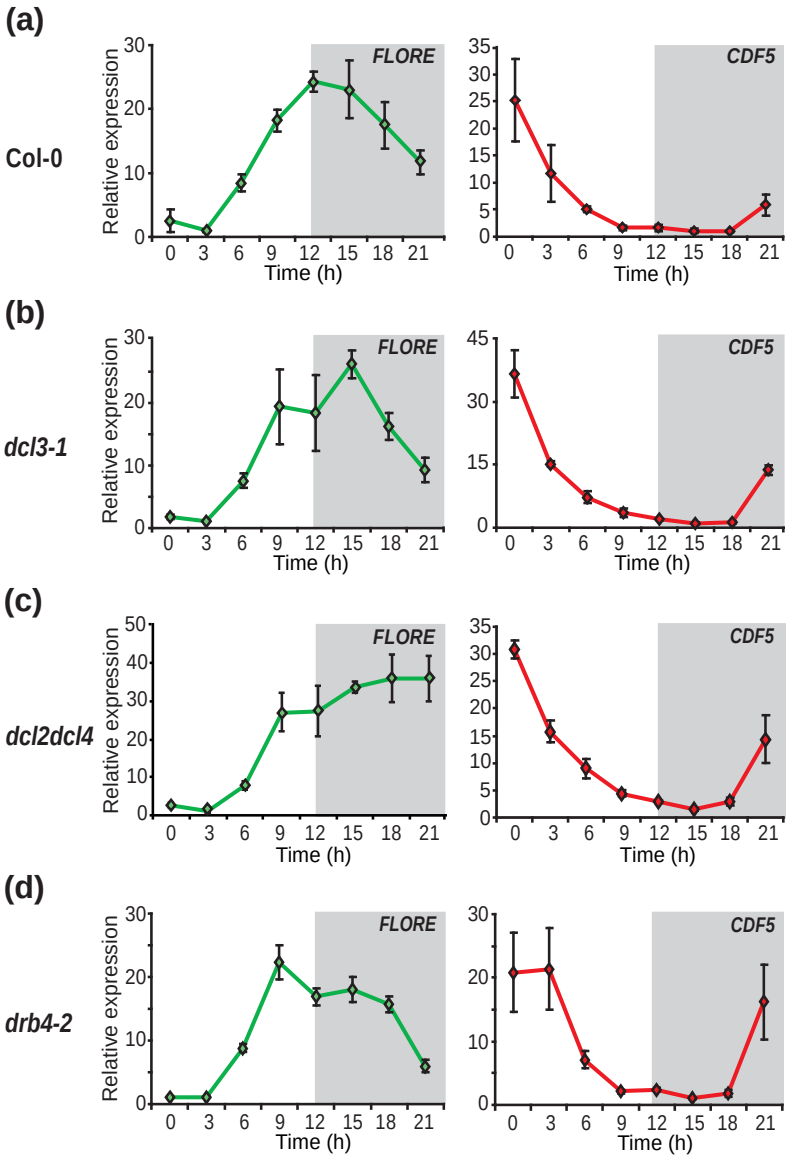


Fig. S5 *FLORE* and *CDF5* expression patterns are conserved in plants affected in siRNA and ta-siRNA biogenesis. *FLORE* and *CDF5* circadian oscillations were determined in WT (Col-0) plants **(a)**; *dcl3-1* **(b)**; *dcl2dcl4* **(c)**; and *drb4-2* **(d)** mutants grown under 12L/D (12 h light / 12 h dark) conditions. qPCR results were normalized with *Actin2* and shown as means ± SD of three technical replicates from one representative experiment out of two biological replicates analysed. Grey rectangles represent the dark period and Time (h) represents hours after lights on.

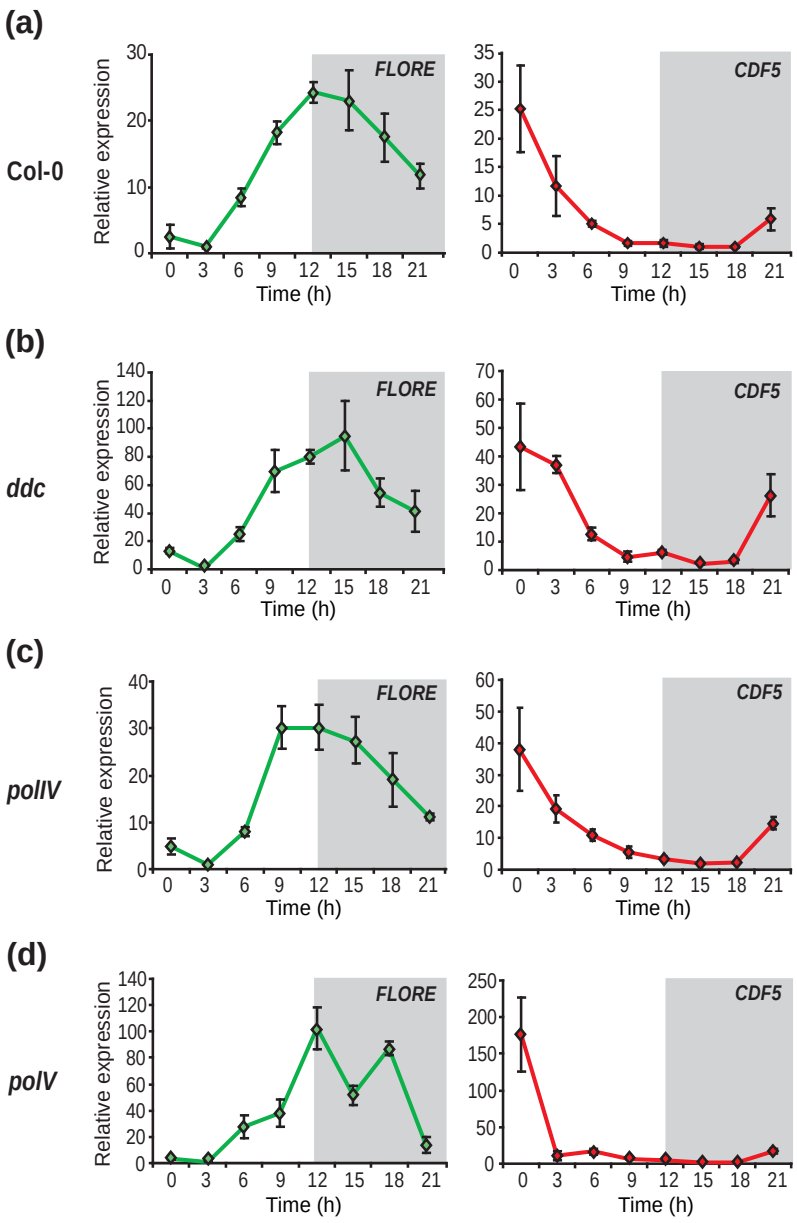


Fig. S6 *FLORE* and *CDF5* waveforms are maintained in plants affected in the RdDM silencing pathway. *FLORE* and *CDF5* circadian expression patterns were determined in WT (Col-0) plants **(a)**; *ddc* (*drm1 drm2 cmt3*) **(b)**; *polIV* **(c)**, and *polV* **(d)** mutants grown under 12L/D (12 h light / 12 h dark) conditions. qPCR results were normalized with *Actin2* and shown as means ± SD of three replicates from one representative experiment out of two biological replicates analysed. Grey rectangles represent the dark period and Time (h) represents hours after lights on.

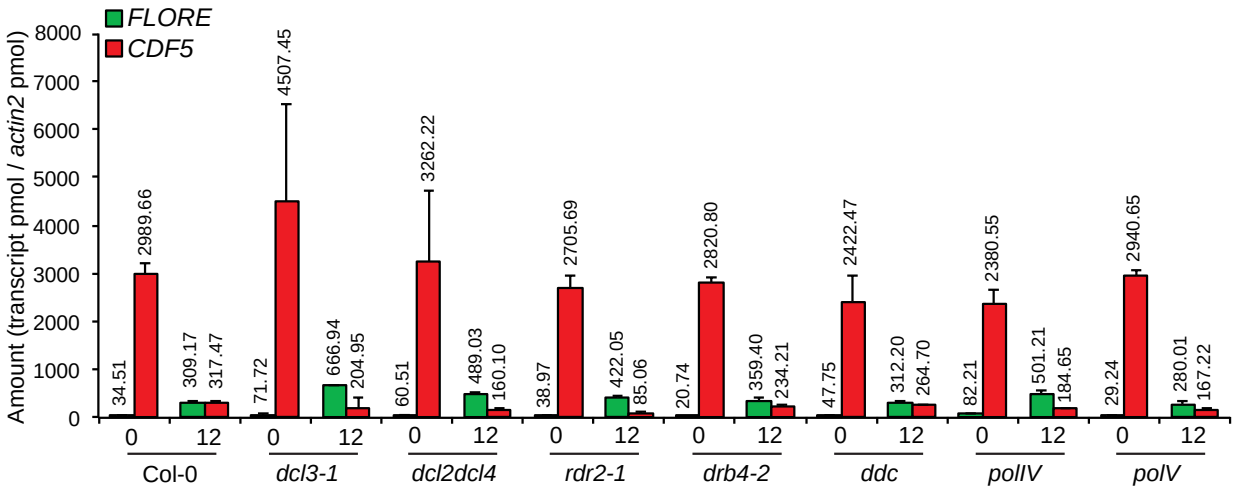


Fig. S7 *FLORE* and *CDF5* transcripts absolute amounts in mutants affected in siRNA or ta-siRNA biogenesis or the RdDM silencing pathway. siRNA biogenesis and RdDM-affected plants evaluated previously under 12L/D conditions were tested for their absolute amounts of *CDF5* and *FLORE* transcripts at their peak times (ZT0 and ZT12, respectively) by qPCR using a fragment-specific standard curve calibration method. These results were obtained after normalization with *Actin2* followed by linear regression analysis of a similar qPCR reaction using standardized amounts of the PCR-amplified specific *CDF5* or *FLORE* fragments. This experiment was repeated twice with similar results. Above the bars are shown the values of transcript (in pmols) after normalization with *Actin2* (in pmols). Red and green bars represent *CDF5* and *FLORE* expression levels, respectively.

Table S1 Primers used for genotyping of *flore-prom*, *cdf5-prom* and *cdf5-5'utr* T-DNA insertion mutants.

Mutant	Primer name	Sequence (5' - 3')
flore-prom (CS812744; Sail-275-A10)	CS812-LP	TATATAACATGAACTTGGGTTGCC
	CS812-RP	GAATATCGAAGCTAGTAAGAGCGC
	LB3 (T-DNA)	TAGCATCTGAATTTCATAACCAATCTCGATACAC
cdf5-prom (SALK_099079)	SALK_099-LP	AAAATGATTATCGGCTTTCAATTC
	SALK_099-RP	TTCAATAGTTATCACGGATTGGTG
cdf5-5'utr (SALK_044252)	SALK_044-LP	TGAAATCTCAGATACTGCTTTTGG
	SALK_044-RP	AACCAAACATTTTTATGTTTTCGG
T-DNA primer	LBb1.3 (T-DNA)	ATTTTGCCGATTTCGGAAC

Table S2 Primers used for cloning of *CDF5* and *FLORE* (genomic, cDNA and promoter sequences).

Construct	Primer name	Sequence (5' - 3')
pENTR-D TOPO:FLORE	TOPO:*FLORE*_Fw	CACCAAATATCTACAGCTCCAAAAATATCTCTGTTT
	TOPO:*FLORE*_Rev	TAACAAATTCAAAGATCCATATATATTATCCGA
	cDNA (+) strand specific p1	TATATTATCCGATCTAAACGAACCA
pENTR-D-TOPO:CDF5 (ORF)	TOPO:CDF5_Fw	CACCATGTCTAAATCTAGAGATACGGAGATAAAGTTG
	TOPO:CDF5_Rev+STOP	TTATTGTTGCATGCTCTCCCTGAAGTT
	TOPO:CDF5_Rev	TTGTTGCATGCTCTCCCTGAAGTTC
TOPO: FLOREprom	TOPO:*FLORE*prom-F	CACCTTAAGGTTCCATAAGATCCACAAGAAAAG
	TOPO:*FLORE*prom-R	CAAACAGAGATATTTTTGGAGCTGTAGATATTT
pBa002a: pFLORE::CDF5-FLAG	*EcoR*V-*FLORE*prom-F	GCCGATATCTTAAGGTTCCATAAGATCCACAAGAAAAGG
	*Aat*II-*FLORE*prom-F	GCCGACGTCCTCTCCATTGTGTGATGAAGTTGCAAC
	*Aat*II-CDF5g-Fw	GCCGACGTCAGCGTGAAAAAGATCTCAAAATGCC
	*Avr*II-CDF5g-FLAG+S-Rev	GCCCCTAGGTTACTTATCATCATCATCCTTATAATCTTGTTGCATGCTCTCCCTGAAGTT

Table S3 Primers used for quantitative real-time RT-PCR (qPCR).

Amplified product	Primer name	Sequence
FLORE (TAIR variant)	ncRNADofL q1_Fw	CCTTCTTCCACAAGACATCGCTGC
	ncRNADofL q1_Rev	TCTAAGTTACCGACAAAACCCGAGAA
FLORE splicing A	ncRNA-splicingA_q1F	TCCACAAGGGTTTGCTTGTAGACTGT
FLORE splicing B	ncRNA-splicingB_q1F	GCTTTGCCTTCTTCCACAAGTTCATAG
FLORE no splicing	ncRNA-No-splicing_q1F	ATTGTTGCATGCTCTCCCTGAAGTT
CDF5	CDF5-q1F	TTTTAACCGGAACCCTGATTGG
	CDF5-q1R	TTTGGGAGGACAATCACATCTCTTT
CDF1	CDF1-q1F	AAGAAGAAGAAAAGAACCAAAACAAGACATTA
	CDF1-q1R	CACCTGAGGTCCAATATCTCTGACAAG
CDF3	CDF3-q1F	CAAATCACCAGAGAAGGTAACTCCAGAG
	CDF3-q1R	CTGCTGATTATCCTCAGGCGTGTC
CO	CO-q3F	CCATTAACCATAACGCATACATTTCATC
	CO-q3R	CCTTGTCTTCCTCTTCTCTCTGTATCTCAG
FT	FT-q4F	TATCTCCATTGGTTGGTGACTG
	FT-q4R	GGGACTTGGATTTTCGTAACAC
Actin2	actin2-q160F	AAGGCCAACAGAGAGAAGATGACTCA
	actin2-q160R	AGAAACCCTCGTAGATTGGCACAGT
IPP2	IPP2-q1F	GTATGAGTTGCTTCTCCAGCAAAG
	IPP2-q1R	GAGGATGGCTGCAACAAGTGT

Methods S1 Hybridization protocol to profile lncRNA expression in *Arabidopsis*.

Briefly, RNA was labelled with Cyanine-3 obtained from 0.5 µg of RNA according to the One-Color Low RNA Input Linear Amplification PLUS kit (Agilent). The labeled RNA sample in 250 µL of 1x Agilent fragmentation buffer and 2x Agilent blocking agent was fragmented at 60°C for 30 min. After mixing with 250 µL of 2x Agilent hybridization buffer the fragmented RNA was subsequently hybridized to the ATH lincRNA v1 array for 17 h at 65°C in an Agilent hybridization oven. Next, microarrays were washed 1 min using GE Wash Buffer 1 (Agilent) and 1 min at 37°C with GE Wash buffer 2 (Agilent). After drying by brief centrifugation, slides were immediately scanned on an Agilent DNA microarray scanner using the default parameters for one colour scan 8 x 15k array slides.

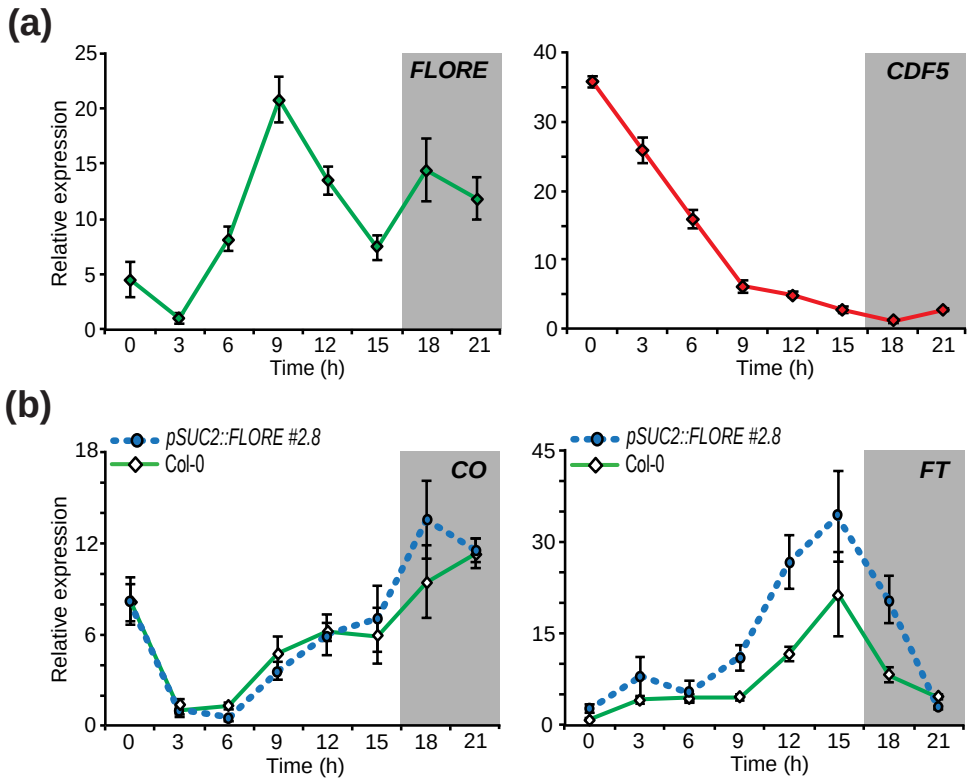
Methods S2 QPCR protocol using fragment specific standard curves.

In the case of the qPCR using fragment specific standard curves, the *FLORE*, *CDF5* and *Actin2* specific qPCR amplified fragments were copied by regular PCR and used as templates in a 10-fold dilution series (0.1, 0.01, 0.001, 0.0001, 0.00001 pg) to determine the linear regression between each transcript amount and corresponding Ct values. Initially the transcript amounts were defined as pg but, in order to compare among fragments of different size (e.g. *FLORE* and *CDF5*) we determined their amounts in moles using the following conversion: average molecular weight of double strand DNA = 660 g/mol. We then calculated the average and standard deviation of sample (either *FLORE* or *CDF5*) and of reference (e.g. *Actin2*). Normalized values were then calculated for each sample by dividing the sample expression average (avg) by the reference gene (*Actin2*) average. The standard deviation (std dev) of this normalized sample was defined as std dev=mean (normalized sample) x CV (coefficient of variation). The CV was calculated as the square root [(std dev/avg of sample, e.g. *FLORE*)^2+ (std dev/avg of reference, e.g. actin)^2]. These values are shown in Fig. S7.

Notes S2 Results from biological duplicates of experiments shown in Fig. 1 and Fig. 5.

Our circadian experiments are presented as "one representative experiment" out of at least two independent biological duplicates with similar results. We present our data in this way since each experiment normally comprises eight independent time points where the oscillation waveform of each genotype is compared to the wild type grown in the exact same conditions. If we combine different biological replicates in one single graph the amplitude of each oscillation varies, and our values due not reflect the actual oscillation pattern of the transcript we analyse.

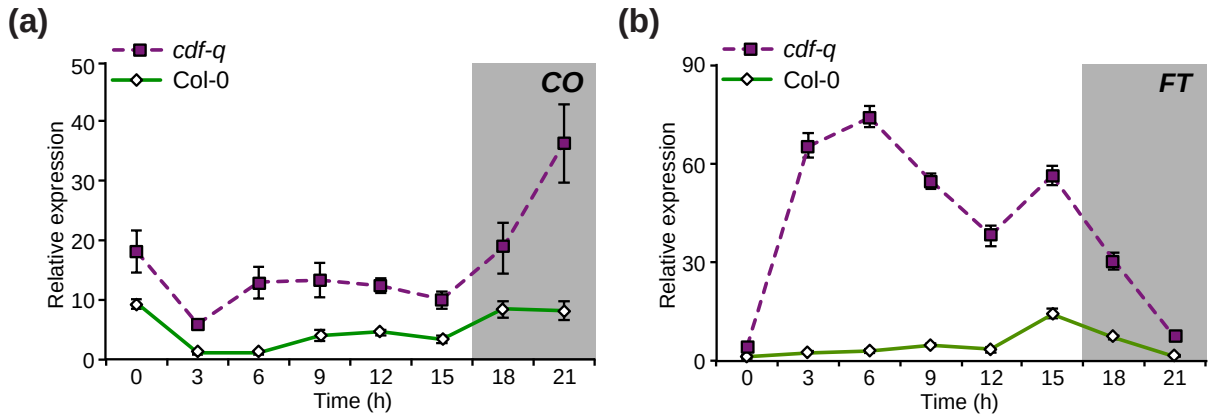
With our approach the oscillation waveform of each transcript shown is representative, for instance the *CDF5* gene is expressed only in the morning whereas its long non-coding RNA *FLORE* peaks always around ZT12, under long day conditions. This is shown in the independent experiments depicted in Figure 1, Figure 2 and Figure 3. In addition, we provide below the results of biological duplicates shown in Fig. 1d and Fig. 5e.



Notes S2 Results from biological duplicates of experiments shown in Fig. 1d and Fig. 5e. *FLORE* and *CDF5* are antiphasic natural antisense transcripts **(a)**. Enhanced expression of *FLORE* in the vascular tissue promotes an increase in *CO* and *FT* transcript levels **(b)**. qPCR analysis was performed as described previously. Grey rectangles represent the dark period and Time (h) represents hours after lights on.

Notes S3 *CO* and *FT* transcript levels in *cdf-q* mutants described in Fig. 2d.

Here we present the transcript levels of *CO* and *FT* in *cdf-q* mutants analysed in Fig. 2d. We found that depletion of *cdf1*, *cdf2*, *cdf3* and *cdf5* promoted higher accumulation of *FT* (3.9-25.8 fold) and a slightly smaller increase of *CO* (1.9-12.6 fold).



Notes S3 *CO* and *FT* transcript levels in *cdf-q* mutants described in Fig. 2d. Wild-type (Col-0) and *cdf-q* seedlings described in Fig. 2d were used to determine the circadian waveforms of *CO* and *FT* transcripts by qPCR after normalization with *Actin2*. Values shown are the means ± SD of three technical replicates from one representative experiment out of two biological replicates analysed. Grey rectangles correspond to the dark period. Time (h) represents the hours after lights on.